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Haemophilus influenzae in children with cystic fibrosis: Antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation

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ABSTRACT

Haemophilus influenzae commonly infects the respiratory tract of patients with cystic fibrosis (CF), early in childhood. In this investigation, 79 *H. influenzae* isolates were recovered from the respiratory secretions of 64 CF patients (median age: 5 years) included in a 5-year follow-up study. Fifteen of the 64 patients contributed two or more *H. influenzae* isolates overtime. Serotyping, antibiotic susceptibility testing, genotyping, detection of both *hmwA* and *hia* adhesin genes and hypermutable strains was carried out. Biofilm formation ability was investigated. Most strains (72/79, 91.2%) were nonencapsulated or nontypeable (NTHi). Resistance to ampicillin (13.9%) and imipenem (17.7%) was the most detected. Few isolates (2.5%) exhibited the hypermutable phenotype. The NTHi strains showed 55 different genotypes, but 19 clusters of closely related strains were identified. Nine clusters included strains that cross-colonised several patients over a long-time period (mean: 3.7 years). Most patients with sequential isolates harboured strains genetically unrelated, but persistent colonisation with the same clone was observed in 37.5% of patients. Over 45% of NTHi strains contained *hmwA*-related sequences, 26.3%, *hia*, 8.3% both *hmwA* and *hia*, while 19.4% lacked both. A significant association was found between occurrence of an adhesive gene (irrespective of which) and both persistence ($P < 0.0001$) and long-term cross-colonisation ($P < 0.0001$). Mean biofilm level formed by the persistent strains was found significantly increased compared to non-persistent ones ($P < 0.0001$). *Hia*-positive strains produced significantly more biofilm than *hmwA*-carrying strains ($P < 0.01$). Although a high turnover of NTHi strains in FC patients was observed, distinct clones with increased capacity of persistence or cross-colonisation occurred.

Introduction

Cystic fibrosis (CF) is a human recessive hereditary disorder caused by mutations in the CF transmembrane conductance regulator gene that regulates transport of electrolytes across the epithelial cell membranes (Tomashefski et al., 1993). Mutations in this gene disrupt electrolyte secretion, leading to a hyper-osmolar viscous mucus and, in the end, to an impairment of the mucociliary clearance function.

As a consequence, the respiratory tract of the CF patients is colonised by pathogenic micro-organisms early in childhood and, in the vast majority of cases, chronic infections are established (Tomashefski et al., 1993; Foweraker, 2009). Recurrent acute respiratory infections together with an aggressive host inflammatory response are thought to play a key role in the irreversible airway damage for which some patients ultimately die (Lyczak et al., 2002). A variety of bacterial pathogens including *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and the *Burkholderia cepacia* complex contributes to morbidity, but the prevalence of the respiratory infections due to the different species change overtime (Harrison, 2007). In infancy, colonisation of the respiratory tract is often initiated by *S. aureus* and *H. influenzae* that can cause acute respiratory infections sustained by either each individual bacterial species or both species (co-infections) (Harrison, 2007; Pettigrew et al., 2008).

It has previously been reported that, among the different *H. influenzae* serotypes, nonencapsulated *H. influenzae* (NTHi) is mostly associated with chronic lung infections and acute exacerbations in CF patients (Murphy et al., 2009a,b). The exposure to multiple antibiotic treatments begins very early in the life of children suffering of CF disease, leading to the possible selection of multiple

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resistant strains, that are difficult to eradicate despite antibiotic therapy. Moreover, NTHi possesses several adherence factors including the HMW1/HMW2 and the *Hia* proteins that may play a role in both successful bacterial colonisation of the human respiratory tract and persistence within it (St. Geme et al., 1993, 1998; Barenkamp and St. Geme, 1996). Since *H. influenzae* has recently been found capable to form biofilms on both middle ear mucosa and airway epithelia, such capability may be a contributing factor to bacterial persistence and disease pathogenesis (Hall-Stoodley et al., 2006; Hall-Stoodley and Stoodley, 2009; Starner et al., 2006).

Whether "*H. influenzae* persistence" is due to permanent colonisation with the same *H. influenzae* clone or subsequent colonisation with different *H. influenzae* clones, is currently the subject of several investigations (Moller et al., 1995; Román et al., 2004; Sá-Leão et al., 2008).

In this study, 79 *H. influenzae* strains isolated from 300 CF pediatric patients, who were included in a 5-year follow-up study, were phenotypically and genotypically characterized in order to (i) study the dynamics of colonisation in each patient (persistence of the same strain vs colonisation with multiple strains) or among different patients (possible presence of cross-colonising clones); (ii) investigate whether *H. influenzae* persistence is associated with either antibiotic resistance or presence of specific adhesins or capability to form biofilm or the sum of these factors.

Materials and methods

Patients and bacterial strains

Three hundred patients (median age 15.4 years), with a history of CF, who were attending the "Bambino Gesù" pediatric hospital in Rome, were included in a 5-year follow-up study (September 2004–September 2009). All patients were screened for *H. influenzae* and other pathogens at three month intervals (once every three months) and at any exacerbation occurrence.

A total of 79 *H. influenzae* strains were recovered from the respiratory secretions (bronchoscopic samples, expectorate sputum and oropharyngeal swabs) of 64 out of 300 patients. Fifteen of the 64 patients contributed two or more *H. influenzae* isolates overtime. At the time of collection of clinical samples positive for *H. influenzae*, patients were visited either at the outpatient department (54 patients) or were admitted to the hospital (10 patients). *H. influenzae* strains were selectively cultured on chocolate blood agar containing Iso-Vitalex and bacitracin (Becton Dickinson, Sparks, USA) and were identified based on the requirement for hemin (X factor) and NAD (V factor) on identification (ID) QUAD Plates (Becton Dickinson). Identification was further confirmed by using the VITEK 2 System (Biomerieux, Marcy l'Etoile-France).

As a control group for the hypermutability study, a sample of randomly selected *H. influenzae* isolates (8 from cerebrospinal fluid and 8 from blood) from non-CF patients was included. This sample was chosen from our collection of *H. influenzae* strains isolated from patients with invasive disease detected through the National Surveillance of Invasive Bacterial Disease (Giufrè et al., 2011). Serotyping was determined by the PCR capsular genotyping, following procedures previously reported (Falla et al., 1994).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, cefotaxime, cefixime, imipenem, azithromycin and chloramphenicol were determined by *E*-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations and using *Haemophilus* Test Medium (HTM). The interpretative criteria were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints/), except for azithromycin, for which the interpretative criteria were adjusted according to the *E*-test protocol for CO_2 incubation (susceptibility breakpoint $\leq$0.250 μg/ml). *H. influenzae* ATCC 49247 was used as control strain. beta-Lactamase activity was detected by the chromogenic cephalosporin test with nitrocefin as the substrate.

Pulsed-field gel electrophoresis (PFGE)

Genetic relationships among the 79 *H. influenzae* isolates were assessed by PFGE after digestion of genomic DNA with SmaI (Roche Diagnostics, Mannheim, Germany) following procedures previously described (Cerquetti et al., 2000), with the only exception of *H. influenzae* type e isolates, for which digestion of plugs was carried out using Apa I (Roche diagnostics) (Cerquetti et al., 2003). Similarity analysis was performed with Dice's coefficient and clustering was carried out by means of the unweighted pair group mean association (UPGMA), with GelCompar II v6.0 (Applied Maths, Sint-Martens-Latem, Belgium). Isolates showing a coefficient of similarity $\geq$80% were considered to belong to the same clonal group or cluster.

Detection of hmw1A, hmw2A and hia genes

The presence of *hmw1A*, *hmw2A* and *hia* adhesion-encoding genes was detected by PCR and confirmed by dot blot hybridization technique. Briefly, PCR analysis was carried out by using primers and following PCR conditions as previously described (Giufrè et al., 2006; Cardines et al., 2007). For dot blot analysis, genomic DNA was spotted onto a nylon membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) and hybridized with *hmw1A*–, *hmw2A*– and/or *hia*-specific probes generated by PCR amplifications of the corresponding genes in prototype strains NTHi12 and NTHi 11, respectively (St. Geme et al., 1998). Positive samples were detected using the ECL system (Amersham Biosciences).

Determination of mutation frequencies

To determine the mutation frequencies, 10 bacterial colonies were re-suspended in 20 ml of Brain Heart Infusion (BHI) broth added with HTM Supplement (Oxoid Ltd., Basingstoke Hampshire, United Kingdom) and grown at 37 °C overnight. Bacterial cells were then collected at 4000 rpm for 10 min and re-suspended in 1 ml of BHI broth. A 100-μl sample from this suspension, as well as samples from successive dilutions, was plated onto HTM with and without rifampin (10 μg/ml). After 48 h of incubation at 37 °C with CO_2, the number of colonies grown on rifampin-containing medium was counted and the mutation frequencies were determined as relative proportions of the total counts of viable organisms plated. A strain was considered to be hypermutable when the mutation frequency was at least 50 times higher than the average mutation frequency found for *H. influenzae* strains isolated from invasive disease. For each hypermutable strain, the experiment was repeated in triplicate and results were indicated as mean value.

Biofilm formation assay

The 79 *H. influenzae* strains were evaluated in sixtuplicate and repeated on two different occasions, for biofilm formation in 96-well culture microplates. Briefly, 200 μl of a standardized inoculum ($2–5 \times 10^6$ CFU/ml) prepared in BHI broth with HTM Supplement (Oxoid Ltd.) was dispensed to each well of a sterile flat-bottom polystyrene 96-well microtiter tissue culture plate (Iwaki, Bibby srl; Milan, Italy) and incubated at 37 °C with 5% CO_2 for 48 h. Quantification of biofilm biomass was then performed by crystal violet assay and expressed as optical density measured at 492 nm

(OD_{492}), as previously described (Pompilio et al., 2008). Strains were classified into the following categories: no biofilm producer ($OD_{492} \leq OD_c$), weak biofilm producer ($OD_c < OD_{492} \leq 2 \times OD_c$), moderate biofilm producer ($2 \times OD_c < OD_{492} \leq 4 \times OD_c$), and strong biofilm producer ($4 \times OD_c < OD_{492}$), where OD_c = mean OD_{492} of control (without inoculum) wells + 3 standard deviations (SDs) (Stepanovic et al., 2007).

Statistical analysis

Biofilm results were statistically evaluated by parametric (unpaired-*t* test) or non-parametric (Mann–Whitney test; Kruskal-Wallis test followed by Dunn's multiple comparison test) tests. Differences between frequencies were assessed by use of the χ^2 test or, when appropriate, Fisher's exact test. Differences with a P value < 0.05 were considered statistically significant.

Results

Detection of H. influenzae and other pathogens associated with CF

Sixty-four out of 300 (21.3%) CF patients included in the 5-year follow-up study harboured *H. influenzae* at least once. The age of the 64 patients ranged from 0.25 to 27 years; mean and median age was 6.5 and 5 years, respectively. Co-colonisation by *H. influenzae* and other bacterial species was present in 42 out of 64 patients (65.6%), including 13 of 15 patients with persistent *H. influenzae* colonisation. Considering the 79 samples positive for *H. influenzae*, 37 (37/79, 46.8%) supported the growth of *Staphylococcus aureus*. The other species more frequently recovered together with *H. influenzae* were: *S. maltophilia* (8/79, 10.1%), *Candida albicans* (5/79, 6.3%), *Klebsiella* spp. (5/79, 6.3%), *Pseudomonas* spp. (3/79, 3.8%) including only one *P. aeruginosa*, enterobacteria (2/79, 2.5%). In 19 samples (24%) *H. influenzae* was the only clinically relevant bacterium isolated.

Of the 79 *H. influenzae* isolates analysed, 72 (72/79, 91.2%) were identified as NTHi, 4 (4/79, 5.0%) were type e, 2 (2/79, 2.5%) were type a and one (1/79, 1.3%) isolate was type f. Considering the 15 patients with sequential *H. influenzae* isolates, 13 harboured only NTHi strains in all repeated samples (irrespective of genetic differences between repeated NTHi strains), one possessed 2 sequential NTHi strains followed by a type e strain and one patient, first colonised by an NTHi strain, harboured a type f strain in the second sample.

Antimicrobial susceptibility

Antimicrobial susceptibility results are shown in Table 1. Resistance to ampicillin and imipenem was the most frequently detected. Among the 79 strains, 11 (13.9%) were ampicillin resistant; of these, 9 were TEM β-lactamase producers (MIC range 16–256 μg/ml) whereas two exhibited a β-lactamase-negative ampicillin-resistant phenotype (BLNAR) with a MIC of 1.5 and 8 μg/ml, respectively. Moreover, 5 isolates showed a slightly decreased susceptibility to ampicillin and were also classified as low-BLNAR (MIC = range 1 μg/ml). Eight strains (10.1%) were found resistant to amoxicillin-clavulanic acid; 4 of these also were β-lactamase-producing strains. No strains were resistant to cefotaxime but 8 strains (10.1%) were cefixime resistant. Fourteen strains (17.7%) were resistant to imipenem (MIC range 3–32 μg/ml), 8 strains (10.1%) were resistant to azithromycin (MIC range 12–256 μg/ml) but 70 strains (88.6%) showed intermediate resistance to the latter antibiotic. No resistance to ciprofloxacin was detected while 3 strains only were resistant to chloramphenicol. Of the 14 imipenem-resistant strains, 5 were β-lactamase producers and 4 were low-BLNAR. Co-resistance between ampicillin, amoxicillin-clavulanic acid, cefixime and imipenem was observed in three strains, one of which showed also resistance to azithromycin.

No significant increase in the prevalence of antibiotic resistance was detected between initial and consecutive *H. influenzae* isolates from the same patient.

PFGE profiles

Of the 72 NTHi strains, 3 were not typeable by PFGE. The remaining 69 NTHi strains yielded 55 distinctive profiles. Overall they exhibited genetic heterogeneity, but 19 minor clusters of genetically closely related isolates (coefficient of similarity ≥80%) were identified, each including from 2 to 5 isolates (Fig. 1, clusters 1 through 19). Twelve NTHi strains were totally unrelated to each other. Of 19 minor clusters, clusters 1, 2, 6, 15 and 16 each included isolates sharing identical PFGE profiles, although they had been isolated in different outpatients and in different time periods. Of note, 9 of the 19 clusters (clusters 4, 10, 12, 13, 14, 15, 16, 17 and 19) included isolates cross-colonising different patients during a long period of time (mean time of 3.7 years) while the remaining 10 clusters (clusters 1, 2, 3, 5, 6, 7, 8, 9, 11 and 18) contained strains isolated within a few months (mean time of 4.1 months). Of 14 patients with at least two sequential NTHi strains, 5 harboured strains with identical profiles suggesting that a persistent colonisation with the same strain or clone had likely occurred, while 9 patients possessed strains without close genetic relationships. Actually, two of these 9 patients each harboured one of the sequential strains not typeable by PFGE (obviously not present in the dendrogram, Fig. 1) but this however suggests the lack of genetic relatedness.

The persistence of the same NTHi clone in the same patient was associated with sample isolation at a brief interval of time (1–6 months) in 4 patients, while the same clone persisted over a period of 2 years in one patient. In this last patient, who contributed with 4 sequential isolates, an indistinguishable strain was isolated in the

Table 1
Antimicrobial susceptibility of 79 *H. influenzae* strains isolated from patients with cystic fibrosis.

Antibiotic	MIC (μg/ml)			Susceptibility category		
	MIC_{50}	MIC_{90}	Range	S (%)	I (%)	R (%)
Ampicillin[b]	0.25	48	≤0.094–≥256	68 (86.1)	–	11 (13.9) [a]
Amoxicillin-clavulanic acid[b]	0.5	1.5	≤0.125–12	71 (89.9)	–	8 (10.1)
Cefixime[b]	0.064	0.19	≤0.012–0.64	71 (89.9)	–	8 (10.1)
Cefotaxime[b]	0.016	0.032	≤0.016–0.094	79 (100)	–	0.0
Imipenem[b]	0.75	12	≤0.125–32	65 (82.3)	–	14 (17.7)
Ciprofloxacin[b]	0.012	0.023	≤0.004–0.5	79 (100)	–	0.0
Azythromycin	3	12	≤0.25–≥256	1 (1.3)	70 (88.6)	8 (10.1)
Chloramphenicol	0.5	1	≤0.25–16	75 (95)	1 (1.2)	3 (3.8)

[a] Nine strains were β-lactamase producers, 2 strains were β-lactamase negative ampicillin resistant (BLNAR).
[b] No intermediate breakpoint.

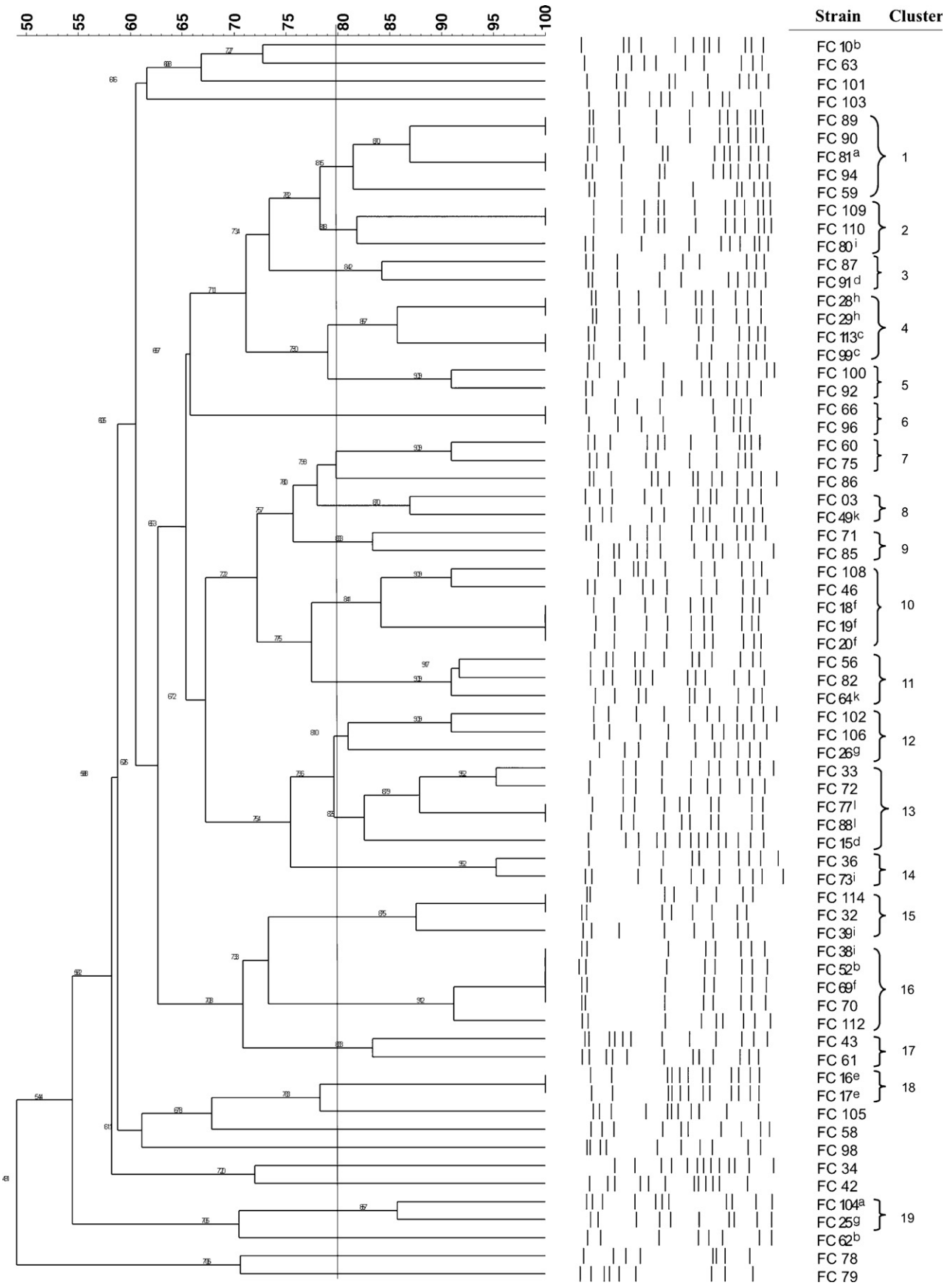

Fig. 1. Genetic relatedness among 69 NTHi strains isolated from CF patients by cluster analysis of the PFGE patterns. Similarity analysis was performed with Dice's coefficient and clustering was carried out by means of the unweighted pair group mean association (UPGMA) method. Strains with a coefficient of similarity value ≥80% were considered to belong to the same cluster. On the right, the strain code number and the different clusters (clusters 1 through 19) are reported. Letters in superscript (a through l) identify patients from which more than one NTHi strain was isolated.

Table 2
Prevalence and distribution of *hmw1A*, *hmw2A* and *hia* genes among 72 ncHi strains.

hia	*hmw1A*	*hmw2A*	No. of strains possessing gene (%)
+	–	–	19(26.3)
+	+	–	4(5.5)
+	–	+	0
+	+	+	2(2.7)
–	+	+	27(37.5)
–	+	–	4(5.5)
–	–	+	2(2.8)
–	–	–	14(19.4)

first three samples over a period of 2 years but a totally unrelated strain was recovered in the fourth sample after 17 months.

The two type a strains isolated from two distinct patients belonged to the same clone (data not shown). Three of the 4 type e strains were genetically closely related (2 showed an indistinguishable profile and one was closely related to them), although isolated from different patients (data not shown). Notably, these clonal type e strains appeared to belong to the major type e clone previously found to circulate among invasive isolates in Italy (Cerquetti et al., 2003).

Adhesins

We evaluated all the 72 NTHi strains for the presence of the genes encoding the HMW and/or HIA adhesins. As shown in Table 2, the *hmw1A*, *hmw2A* or *hia* genes were distributed among our strains in various combinations. In particular, a high number of the NTHi strains (27/72, 37.5%) contained sequences for both *hmw1A* and *hmw2A*. Six strains were found positive for either *hmw1A* (4 isolates) or *hmw2A* (2 isolates). Nineteen strains (19/72, 26.3%) were *hia* positive. A few strains possessed *hia* together with either *hmw1A* alone (4/72, 5.5%) or *hmw1A* and *hmw2A* (2/72, 2.7%). Notably, a relatively high number (14/72, 19.4%) of strains lacked both *hmwA* and *hia*-related sequences. Among the 5 persistent NTHi clones isolated from the same patients with sequential isolates, 3 carried *hia* while 2 possessed both *hmw1A* and *hmw2A*.

Detection of hypermutable strains

The mean mutation frequency of the 79 *H. influenzae* isolates from the CF group (9.1×10^{-9}) did not significantly differ from that of the non-CF group (3.0×10^{-9}) ($P = 0.241$, Mann–Whitney U test). However, two isolates (2/79, 2.5%) from the CF group were clearly distinguishable, since they exhibited a hypermutable phenotype with a mutation frequency of 3.4×10^{-7} (strain FC 74) and 2.1×10^{-7} (strain FC 13), respectively. Both these strains were found to be strong biofilm producers with strain FC 74 showing the highest production ($OD_{492} = 0.999$) among all the 79 *H. influenzae* isolates. None of the two strains showed resistance to the antimicrobial agents tested, with the only exception of resistance to imipenem exhibited by FC13.

Biofilm formation

We examined and compared the biofilm formation of the 79 *H. influenzae* isolates on plastic surfaces by using 96-well polystyrene microtiter plates. All *H. influenzae* isolates tested were able to form biofilms, although levels of production varied greatly among them, ranging from an OD_{492} of 0.176 (strain FC 3) to 0.999 (strain FC 74). As regards biofilm categories, interestingly, a significantly higher percentage of strong biofilm producers was found compared to moderate biofilm producers (63.2% vs 36.8%, respectively, $P < 0.001$, Fisher's exact test). No weak biofilm producer strains were detected. There was no significant difference in mean

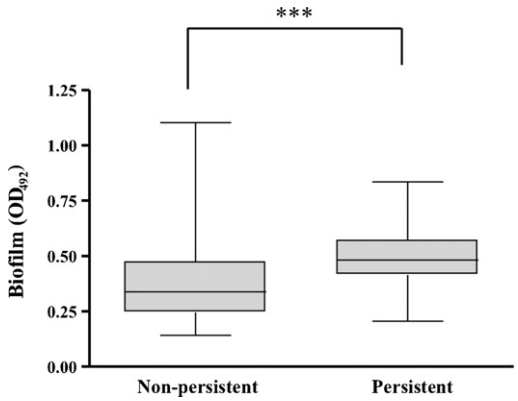

Fig. 2. Biofilm formation on polystyrene by 79 *H. influenzae* strains categorized as "non-persistent" and "persistent". Isogenic strains with indistinguishable PFGE profiles sequentially (at least twice) isolated from the same patient with at least one month interval were classified as "persistent", all other strains (strains isolated only once in a patient and/or replaced by unrelated strains with different PFGE profiles) were "non-persistent". Each strain was tested in sixtuplicate on two different occasions. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median (50th percentile); the whiskers indicate the lowest and the highest value. ***$P < 0.0001$, Mann–Whitney test.

biofilm level between strains cross-colonising different patients over a long time period and strains cross-colonising patients over a short time period (data not shown). Concerning sequential isogenic strains isolated from the same patient (5 clones including 11 strains isolated from 5 patients), the mean biofilm level formed by the 11 persistent strains was significantly higher compared with that produced by all the other strains analysed in this study ($P < 0.0001$), suggesting that persistence of a distinct strain within a patient is associated with high biofilm-forming ability (Fig. 2). However, looking at each of the 5 persistent clones, significant differences were found among isogenic strains from the same patients. In particular, a significant increase in biofilm formation was observed over time between the two sequential isogenic strains in 3 patients (mean $OD_{492} \pm$ SD: 0.522 ± 0.03 vs 0.755 ± 0.042, for strains FC 28 and FC 29, respectively, $P < 0.0001$; 0.243 ± 0.023 vs 0.561 ± 0.026 for strains FC 77 and FC 88, respectively, $P < 0.0001$; 0.246 ± 0.013 vs 0.417 ± 0.015 for strains FC 16 and FC 17, respectively, $P < 0.0001$), a significant decrease was detected in one patient although the level of biofilm production remained high (mean $OD_{492} \pm$ SD: 0.695 ± 0.096 vs 0.529 ± 0.080 for strains FC 99 and FC 113, respectively, $P = 0.009$) and a significant rise followed by a significant decrease in the remaining patient contributing with three sequential isogenic strains (mean $OD_{492} \pm$ SD: 0.475 ± 0.024 vs 0.569 ± 0.018 vs 0.439 ± 0.022, for strains FC 18, FC 19 and FC 20, respectively; $P < 0.01$). The role played by *H. influenzae* adhesins in biofilm formation was also assessed (Fig. 3). Strains positive for *hia* formed significantly more biofilm than *hia*-negative ones (mean $OD_{492} \pm$ SD: 0.463 ± 0.126 vs 0.353 ± 0.150, respectively; $P < 0.01$). On the contrary, the presence of *hmw1A* or *hmw2A* significantly reduced biofilm levels, both considered individually (mean $OD_{492} \pm$ SD; 0.356 ± 0.150 vs 0.430 ± 0.143, and 0.350 ± 0.152 vs 0.423 ± 0.143, for *hmw1A+* vs *hmw1A–*, and *hmw2A+* vs *hmw2A–*, respectively; $P < 0.05$) and in combination (mean $OD_{492} \pm$ SD: 0.349 ± 0.153 vs 0.434 ± 0.141, for *hmw1A+/hmw2A+* vs *hmw1A–/hmw2A–-*, respectively; $P < 0.05$). Comparing strains possessing *hia* with those containing *hmw1A* or *hmw2A*, or both (irrespective of the different combination of *hia* and *hmwA* genes in each isolate) the *hia*-positive strains formed significantly higher levels of biofilm (mean $OD_{492} \pm$ SD: 0.463 ± 0.126 vs 0.356 ± 0.150, 0.350 ± 0.152, or 0.349 ± 0.153, respectively; $P < 0.01$) (Fig. 3), thus confirming the critical role played by *hia* in biofilm formation. When we considered the seven

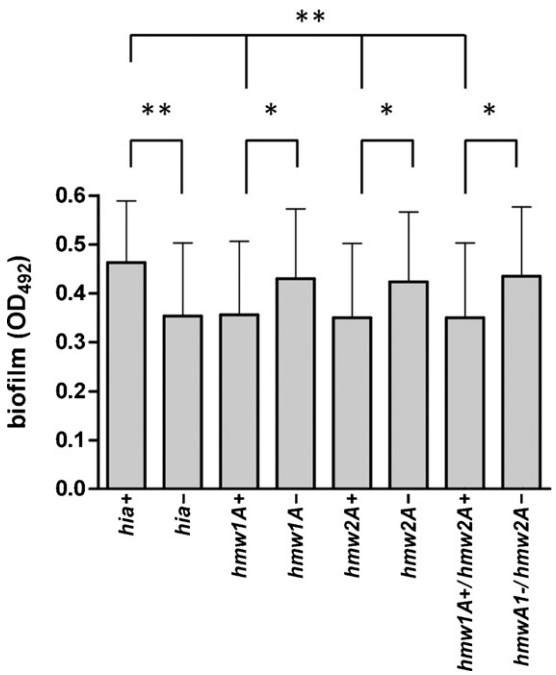

Fig. 3. Biofilm formation on polystyrene by 72 *H. influenzae* strains according to the presence of genes encoding *Hia*, HMW1A and/or HMW2A adhesins. Each strain was tested in sixtuplicate on two different occasions, and results were expressed as means + SDs. $^{*}P < 0.05$ and $^{**}P < 0.01$, unpaired-*t* test.

adhesin genotypes resulting from the different combinations of *hia*, *hmw1A*, or *hmw2A* genes (see Table 2), no statistically significant differences in biofilm level were found among genotypes, including strains lacking both types of adhesins (*hia*$^{-}$/*hmwA1*$^{-}$/*hmwA2*$^{-}$) (data not shown).

Overall, our results suggest that strong biofilm producers were associated with the presence of *hia* rather than with a specific adhesin genotype and that factors other than *hia* can also play a role in biofilm development.

Finally, resistance to azythromycin, ampicillin, or imipenem did not significantly affect biofilm formation efficiency (data not shown).

Discussion

In our cohort of patients, the prevalence of *H. influenzae* was 21.3%, in agreement with previous investigations showing a similar proportion of *H. influenzae* colonisation/infection among pediatric patients (Saiman, 2004; Harrison, 2007; Razvi et al., 2009). Co-colonisation was present in the majority of the patients harbouring *H. influenzae*. According to their young age, *S. aureus* was the most frequently simultaneous pathogen. On the contrary, *Pseudomonas* spp., that is the most relevant pathogen in adults with CF, was very rare. Interestingly, *S. maltophilia* was isolated concomitant with *H. influenzae* in a substantial percentage (10.1%) of CF patients (mean age: 10.3 years) in agreement with several recent studies reporting that the incidence of *S. malthophilia* infection in CF has currently increased especially among adolescents (Razvi et al., 2009; Lipuma, 2010).

As expected, most *H. influenzae* strains characterized in this study were NTHi and, among encapsulated strains, few type a, type e and type f strains were detected. To our knowledge, this is the first time that type a strains have been isolated in Italy, whereas type e and f strains have previously been found to circulate among invasive *H. influenzae* isolates (Cerquetti et al., 2000, 2003, 2004).

The wide use of aggressive antibiotic treatments in the therapy of CF disease may result in an increasing risk of emergent antibiotic resistance, as demonstrated in pathogens such as *P. aeruginosa* and *S. aureus* (Besier et al., 2007; Merlo et al., 2007). In *H. influenzae*, little is known on antibiotic resistance trends in CF patients (Möller et al., 1998; Román et al., 2004). Overall, we did not observe a very high degree of antibiotic resistance. Besides azithromycin (for which EUCAST breakpoints have been set to categorize wild-type *H. influenzae* as intermediate), the majority of the strains (69.6%) were fully susceptible to all antibiotics tested and only one patient was colonised by a multi-drug resistant (non-susceptibility to ≥3 structurally unrelated drugs) strain. However, a rising emergence of imipenem resistance was detected in comparison with previous data on invasive *H. influenzae* isolates (Giufrè et al., 2011). Interestingly, some imipenem-resistant strains showed susceptibility to ampicillin, although with increased MIC (MIC = 0.5 μg/ml). We did not investigate the molecular basis of the imipenem resistance in our CF strains, however, a previous study of ours demonstrated that imipenem resistance phenotype depends on a specific pattern of amino acid substitutions in penicillin-binding protein 3 and that this pattern is unable to confer full resistance to ampicillin, although it affects the level of susceptibility (Cerquetti et al., 2007). Notably, no association was found between increase in antimicrobial resistance and persistence of the same strain in the same patient. This finding should be discussed taking into account that most strains herein described were found to be strong biofilm producers and bacterial cells in biofilms might be recalcitrant to antibiotic treatment despite demonstrated susceptibility of planktonic bacteria (Walters et al., 2003; Hall-Stoodley et al., 2009; Høiby et al., 2010).

High frequencies of hypermutable strains have previously been reported for pathogens in the CF setting, including *H. influenzae*, but our data did not confirm this observation (Román et al., 2004; Watson et al., 2004). Actually, since the link between hypermutation and antibiotic resistance (i.e. mutators are more frequently antibiotic-resistant) (Oliver, 2010), the low prevalence of hypermutators is in agreement with the moderate rate of antibiotic resistance we detected. Besides antibiotic resistance, hypermutation has also been linked to other genetic adaptive advantages enabling bacteria to better survive in airways of CF patients (Mena et al., 2008; Oliver and Mena, 2010). Recent studies showed that genetic exchange occurs in mono- and polymicrobial biofilms, by both conjugation and transformation, thereby expanding the *in vivo* genetic pool and increasing the likelihood that hypermutators will arise (Ghigo, 2001; Molin and Tolker-Nielsen, 2003). In this view it is noteworthy that the strain FC 74 exhibiting the highest mutation frequency was also found to be the highest biofilm producer. Since the majority of hypermutable bacteria including *H. influenzae* have been found to have defects in some components of the methyl-directed mismatch repair system, particularly *mutS* gene, we investigated the latter in our two hypermutable strains, following procedures previously described (Watson et al., 2004; Pérez-Vázquez et al., 2007). In spite of the use of several primer sets, PCR amplification of the *mutS* gene failed in strain FC 13, suggesting that either a wide rearrangement or even a possible deletion of the gene occurred (data not shown). On the contrary, a large amplicon of 2232 bp in length was obtained from strain FC 74, although we were unable to amplify the whole *mutS* gene (data not shown). The partial MutS amino acid sequence showed quite a degree of polymorphism in comparison with the *H. influenzae* Rd KW20 (Fleischmann et al., 1995) with the presence of 17 amino acid substitutions (EMBL database accession no. HE576768). Whether these mutations in *mutS* are responsible or not for the hypermutable phenotype we observed requires further investigations.

The extensive genetic diversity of the NTHi population has been well described in the literature (Smith-Vaughan et al., 1998;

Meats et al., 2003). Our investigation of the genetic relationships among NTHi isolates from CF patients only partially confirmed such high heterogeneity, since numerous clusters of related genotypes were identified, although each including only a few isolates. We could speculate that the lesser heterogeneity among FC isolates we observed might result from selection within the CF airways of definite genotypes well adapted to the particular environment encountered. A limited patient-to-patient transmission may be supposed for cross-colonising strains isolated from different patients within a short period of time, but little evidence for a direct contact between patients was found (most patients involved were visited at the outpatient department). Concerning the patients with sequential *H. influenzae* isolates, the initial NTHi strain was subsequently replaced by another strain (NTHi or an encapsulated strain) in the majority of patients, confirming previous results that *H. influenzae* colonisation is a dynamic process (Román et al., 2004). However, since we did not investigate several colonies from the original growth culture, we cannot exclude that some patients might harbour more than one *H. influenzae* strain and that we failed to isolate the initial strain in the following samples or vice versa. Certainly, persistence of the same NTHi clone was observed in more than one third of patients (35.7%), suggesting that a definite strain may be particularly fit for colonisation/infection in CF patients.

To investigate what makes strains fit for colonisation and persistence we analysed the occurrence of the adhesive genes among our NTHi strains. Although the majority of strains contained *hmwA*-related sequences, irrespective if they were positive for *hmw1A* or *hmw2A* or both, the percentage we found was lower than that previously reported, for NTHi clinical isolates from different sources, in the literature (45.8% vs 70–80%) (St. Geme et al., 1993, 1998). On the contrary, the proportion of the *hia*-positive strains was slightly higher in comparison with previous data (26.3% vs 15–20%) (Barenkamp and St. Geme, 1996; St. Geme et al., 1998). Interestingly, we noticed that all persistent strains as well as most long-term cross-colonising strains (81.3%) possessed either *hmwA* or *hia* genes or both. A statistically significant association between the occurrence of an adhesin gene, irrespective of which, and both persistence ($P < 0.001$) and long term cross-colonisation ($P < 0.001$) was found, underlining the importance of possessing adhesive properties in order to be a "successful" strain in CF patients. Structures suggestive of NTHi biofilm formation have been recently observed in the bronchoalveolar lavage fluid of young asymptomatic patients with CF (Starner et al., 2006). Here we investigated biofilm formation by using a quantitative assay on plastic surface and our results confirmed that ability to form biofilm is a crucial feature of pathogens isolated from CF patients (Hall-Stoodley and Stoodley, 2009). A remarkable strong correlation was found between high levels of biofilm production and persistence of the same strain in the same patient. This finding is particularly interesting and it is consistent with previous considerations that bacteria in biofilm may be more difficult to eradicate as they can resist to both the host immune system and antibiotic treatments (Vuong et al., 2004; Shah et al., 2006; Hall-Stoodley and Stoodley, 2009). However, despite the mean burden of biofilm associated with our persistent strains was high, significant differences in biofilm levels among isogenic strains isolated from the same patient were observed, suggesting that not only the strain genotype is critical for *H. influenzae* biofilm formation. Since each persistent strain was sequentially isolated from the same patient at different times, we speculated that phenotypic variations in biofilm forming ability might occur during the different stages of infection, enabling *H. influenzae* to survive the different environmental conditions and stresses encountered. Previous studies on other pathogens such as *P. aeruginosa* have indicated that biofilm development is a sequential process that involves different steps including initial attachment of bacteria (Klausen et al., 2003). For the first time in *H. influenzae*, we described a positive correlation between a specific adhesin (the *Hia* adhesin) and biofilm levels, suggesting that *Hia* may play a role in the development of biofilm. On the contrary, an inverse relationship between HMW adhesins and biofilm was evident. It is intriguing to note that, as mentioned above, the occurrence of the *hmwA* genes in our CF strains was strongly reduced. A limitation of our study was that we looked for the presence of *hia* and *hmwA* genes but no investigations on the actual level of *Hia* and HMW proteins expression were carried out. Previous results from other authors demonstrated that the actual expression of the *hmw1A* and *hmw2A* genes may be modulated during the different phases of disease by a phase variation mechanism (Dawid et al., 1999). Decreased quantities of HMW1 and HMW2 adhesins were found to be expressed, over time, in serial *H. influenzae* isolates from patients with chronic obstructive pulmonary disease and associated with high serum titers of HMW1/HMW2-specific antibodies, suggesting that selection against organisms expressing high levels of HMW proteins might allow *H. influenzae* to persist in the lower respiratory tract of these patients (Cholon et al., 2008). Our results are in agreement with this last observation indicating that strains carrying *hmwA* genes did not seem to be positively selected in the environmental conditions of the CF patients.

The specific role that *H. influenzae* plays for the development of the CF lung disease is still a matter of debate. Interference and commensalisms with other possibly present pathogens should be considered, since the importance of the microbial communities has been recently underlined (Harrison, 2007). Data from the present investigation showing that more than 20% of the young CF patients were colonised by *H. influenzae* (together or not with other microbial pathogens) suggest that this microrganism may contribute to the local inflammatory response and therefore to the early damage of the lung. A recent approach in the therapy of CF disease involves the use of aggressive prophylactic administrations of antibiotics with the aim to delay or prevent chronic colonisation. Although there is no evidence that colonisation by *H. influenzae* severely worsens prognosis of disease, such preventive practice may be considered in patients suffering for repeated acute exacerbations involving *H. influenzae*. However, since *H. influenzae* isolates from CF patients have been found to be strong biofilm producers, some kind of tolerance to antibiotics might occur *in vivo*. Further investigations are needed to clarify this item.

In conclusion, colonisation of the respiratory tract with *H. influenzae*, especially NTHi, was found to be quite frequent in young CF patients. Despite recurrent antibiotic therapy, a moderate rate of antibiotic resistance was overall observed among *H. influenzae* isolates from CF. However, a rising emergence of resistance to imipenem was documented. Numerous genetically diverse NTHi clones were present and colonisation with multiple clones was observed in the majority of patients. However, some selection of the fittest strains with increased capacity of either persistence or cross-colonisation had likely occurred. Presence of an adhesive gene seems to be associated with both properties while a particularly high ability to form biofilm mainly contributes to strain persistence. *Hia* adhesin may play a role in the development of such a biofilm structure.

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